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TITLE: Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness

PRINCIPAL INVESTIGATOR: Dr. Mohamed Abou-Donia

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, NC 27710 US

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14. ABSTRACT The purpose of this project is to develop objective peripheral biomarkers for Gulf War Illness (GWI). We planned to determine circulating autoantibodies to ten proteins associated with the central nervous system in sera and plasma from a group of 250 Gulf War veterans with Gulf War Illness (GWI) and from 200 controls (100 healthy GW controls, 100 disease controls). Preliminary results using Western blot assay showed increased levels of autoantibodies in GWI cases compared to chronic fatigue syndrome, irritable bowel syndrome and low back pain controls. These results confirm continuing presence of autoantibodies against neural proteins in GW veterans and are in agreement with recent reports indicating that 26 years after the war, the health of veterans with GWI is not improving. Such blood-based autoantibodies may prove useful as biomarkers of GWI, upon validation of the					
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1. Introduction

The Subject: Assaying of autoantibodies against neuronal and glial proteins in veterans with Gulf War Illness (GWI) using Western blot and ELISA assays. **PURPOSE:** Development of peripheral biomarkers for GWI. **Scope of the Research:** Serum and plasma from 250 Gulf War veterans with GWI and 200 controls (100 healthy GW veterans, 50 chronic fatigue syndrome (CFS) and 50 irritable bowel syndrome (IBS) will be included in the study.

2. Key Words

Gulf War Illness (GWI) Microtubule Associated Proteun-2 (MAP-2), tubulin, Neurofilament proteins (NFP), tau myelin basic protein (MBP), Myelin Associated Glycoprotein (MAG), CaMKII, alpha-synuclein, GFAP, S100B, Western Blot, ELISA, chronic fatigue syndrome (CFS), irritable bowel syndrome (IBS)

3. Accomplishments

- What were the major goals of the project?
 - The major goals of the project as stated in the approved SOW are listed in the table below. Milestones/target dates for important activities or phases of theses dates and actual completion dates are listed in the table below.
- What was accomplished under these goals?
 - The table lists the proposed goals and dates to accomplish and the actual dates that these goals were accomplished during the first 24-month period

Tasks	Timeline	
Task 1: Obtain Regulatory Reviews and Approvals	Planned Months	Actual Months
1a. Obtain necessary IRB approvals or Exempt status	1-3	1-4
1b. Obtain DOD Human Research Protections Office (HRPO) approvals or Exempt Status	1-3	1-4
Milestone(s) Achieved: Regulatory reviews completed and final approval obtained for study	1-3	1-4
Task 2: Obtain Stored Blood Serum and CSF samples from 3 biorepositories for analysis.	Months	
Proposed 2a: 100 GWI and 50 IBS serum samples shipped from Site 5 to Duke and NIH for analysis.	4-6	
<i>Actual 2 a:</i> 100 GWI and 50 IBS plasma samples shipped from Site 5 to Duke and NIH for analysis		6-8
<i>Actual 2a:</i> Adapting western blot for plasma samples		10-11
<i>Actual 2a:</i> Beginning analysis of all plasma samples from 100 GWI and 50 controls.		10-12

Proposed 2b: 50 GWI and 50 healthy GW veteran controls and 50 CFS control serum samples shipped from site 4 to Duke and NIH for analysis. Actual 2b: Plasma samples received from NOVA University: 1. 50 CFS samples (site 4) 2. 26 healthy GW control samples (site 4) 3. 68 GWI samples from site 1 (Boston GWIC)	4-9	12
4. 50 IBS samples from site 5 (BIDMC) 5. Gulf War Illness (GWI) – 100 samples 6. Irritable Bowel Syndrome (IBS) - 50 sample	10	14
*Proposed 2c: 100 GWI and 50 healthy GW veteran control serum samples shipped from site 1 to Duke and NIH for analysis.	10-22	
Actual 2c: Plasma samples from Site 4 (NOVA) Received were: Gulf War Illness(GWI) – 68 samples (Total GWI (68 + 100 = 168) Chronic Fatigue Syndrome (CFS) – 50 samples Healthy Control – 26 samples	12	16
Proposed *2d: 25 GWI and 25 healthy GW veteran control CSF samples shipped from site 1 to NIH for analysis	22-24	
Actual 2d: CSF samples still being collected at site 1 so not shared yet.		
Milestone(s) Achieved: Site 1, 4 and 5 serum and CSF data collected and set up for laboratory assays (ELISA, western blot). Autoantibody data shipped to analyzing labs from 250 GWI veterans and 200 controls (100 healthy and 100 diseased controls) blood serum samples and 50 CSF (25 GWI, 25 control) samples.	3-24	Samples were shipped to Duke lab for analysis from three sites including 294 samples (168 GWV; 50 IBS; 50 CFS)
Task 3: Perform Serum Assays	Months	
3a: Perform western blot analyses for autoantibodies to CNS proteins in GWI cases and control samples.	4-24	22
3b: Perform ELISA analyses for Neurofascin 155 CNS marker in serum samples from GWI cases and controls.	3-24	

Milestone(s) Achieved: Autoantibodies for CNS proteins of myelinogenesis, astrogliogenesis and neurogenesis data analyzed from three biorepository sites.	9-24	294 autoantibodies run for 9 CNS markers. Currently being merged with demographic datasets for further analysis.
Task 4: Perform CSF Assays	Months	
4a: Perform ELISA assays of 50 CSF samples for neurofascin 155 biomarker.	22-24	Not completed yet because CSF still being collected at site 1.
4b: Merge CSF outcome data with clinical neuroimaging, TBI and exposure data.	24-27	
Milestone(s) Achieved: Antibody for neurofascin 155 marker data analyzed and merged with clinical outcome data from GWIC biorepository site.	24-27	
Task 5: Merge Data and Perform Interim Data analyses	Months	
Proposed 5a: Merge clinical dataset from sites 1, 4, 5 case/control status and demographics with results from laboratory analyses performed at NIH and Duke. Actual 5a: Case/control status merged with autoantibody results for GWI, CFS, IBS groups. Abstracts submitted. Merging with other demographics still being conducted.	10-24	18-24
Proposed 5b: Data analysis of interim ELISA and western blot results of autoantibodies in GWI cases and controls (healthy and diseased groups) with merged clinical datasets. Actual 5b: Merging is still ongoing with clinical datasets.	18-24	
Proposed 5c: Discussion of results and preparation of abstracts for presentations at national meetings and initial manuscript for publication. Actual 5c: In person meeting held in Boston on August 23, 2007 where results, presentations and abstracts were planned. Third year plans were discussed.	18-24	22 A meeting held in Boston University on August 23, 2007.

<p>Proposed 5d. Annual reports of progress will be written.</p> <p>Actual 5d. Two yearly progress reports submitted on October 23, 2017.</p>	12-24	12, 24
<p>Milestone(s) Achieved: Preliminary analysis of results and presentation of initial results at scientific meetings and potential publication.</p> <p>Actual: 3 abstracts accepted for scientific meetings, 1 paper published to date, two more in preparation.</p> <p>Possible biomarker selection for GWI and recommendations for treatment development.</p>	18-24	18-24
<p>Milestone Achieved:</p> <ul style="list-style-type: none"> ○ <i>Determination of autoantibodies against neural proteins in veterans of the Gulf War who have Gulf War Illness (GWI), GW controls, CFS and IBS symptomatic controls.</i> 		
<p>Milestone Achieved: <i>Development of ELISA assay for all neural proteins that we assay as biomarkers for nervous system injury</i></p>		
Task 6: Perform Final Data analyses and Prepare Manuscripts for Publication	Months	
6a: Merge clinical datasets for neuroimaging, blood and genetic biomarkers, brain injury and exposure history with GWI cases and controls.	25-30	
6b: Perform Data analysis comparing ELISA and western blot autoantibodies outcomes in GWI cases and controls with merged clinical datasets for neuroimaging, blood and genetic biomarkers, brain injury and exposure history.	25-30	
6c: Discuss results of data analyses and prepare abstracts for DOD and other scientific meetings.	25-32	
<p>6d: Preparation of manuscripts</p> <ul style="list-style-type: none"> • Diagnostic CNS Autoantibody Biomarkers for GWI • Biomarkers for Gulf War patients with chronic fatigue syndrome (CFS) and others with Irritable Bowel Syndrome (IBS), OP pesticide and nerve agent exposures in GW veterans • Biomarkers of prior brain injury in GW veterans against their combination, 	25-36	

6e: Final report of progress will be written.	35-36	
Milestone(s) Achieved: Analysis of all study results, presentation of results at scientific meetings, submitted publication and final report in progress. Possible diagnostic biomarker selection for GWI, brain injury and deployment-related exposures and potential recommendation for treatment development.	35-36	

*** Serum and Cerebrospinal Fluid (CSF) samples will be collected as part of the ongoing Boston GWI consortium study and will be sent to NIH and Duke study sites as the samples are added to the GWIC biorepository. These samples will all be collected by month 24 of the current study.**

- **What was accomplished under these goals?**

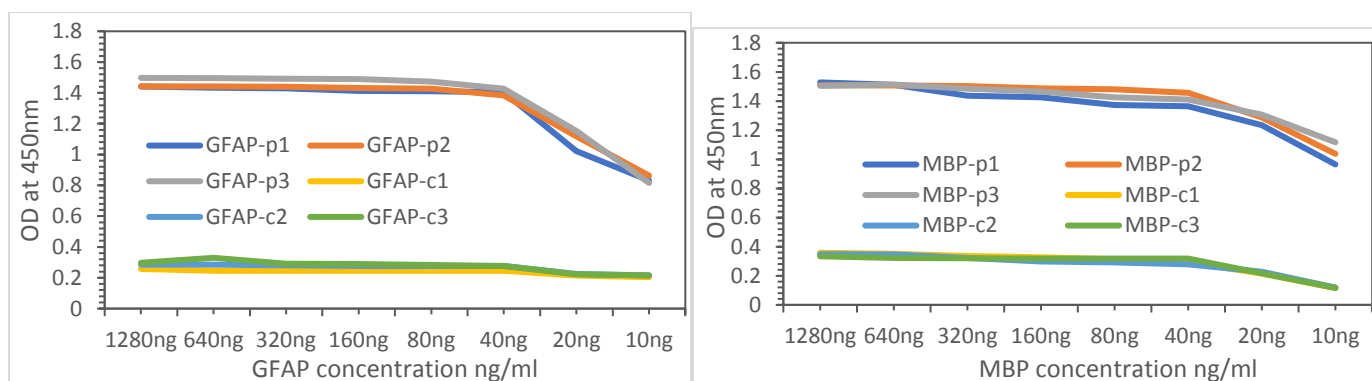
- *Major activities:* **Sending blood samples from nearly 300 GWV and symptomatic controls from three study sites to Duke Laboratory for analysis.** It was determined during early discussions with other co-investigators that more plasma than serum was available at the study sites. So a large aim of this period was to adapt the Western blot assay of autoantibodies from serum samples to plasma samples that way both could be used in this study and importantly in other validation studies and clinical assessments in the future.
- *Determination of autoantibodies against neural proteins in veterans of the Gulf War who have had Gulf War Illness (GWI), GW veteran healthy controls, Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS) symptomatic control groups have been completed.*
- *Development of ELISA assay for all neural proteins that we assay as biomarkers for nervous system injury.*
- *Specific objectives:*
 - We established Western blot analysis for autoantibodies against neural proteins in plasma samples from GWI subjects and from controls similar to our original method in serum samples from a small pilot study.
 - Quantitative measurement of neuronal autoantibodies by ELISA in the sera of patients with Gulf War Illness (GWI).
- *Significant results:* These results now allow performing autoantibody assays not only in serum but also in plasma. There is a considerable advantage because many specimens from GW veterans are collected and processed as plasma not serum and other stored samples could now be used for this purpose and repeated by other laboratories.
- *Other achievements.* Currently, we have adapted our autoantibodies assays against neural proteins to ELISA assays. This will give another dimension in assaying large number of samples to these biomarkers. Furthermore, the development of ELISA assay for autoantibodies will allow faster performance of assays and provide more quantitative results that are easily reproducible.
- In addition to GWI, some veterans also have other conditions such as CFS and IBS. We carried out a study to compare biomarkers for GWI, CFS and IBS. The results showed that patients with CFS and IBS had lower levels of autoantibodies (AA) against fewer neural proteins, indicating that the levels of AA against neural proteins in these patients were lower than those seen in veterans with GWI. This suggests that GWI can be distinguished from CFS and IBS groups if this finding is replicated in the larger study sample.

- **Development of ELISA Method to detect neural autoantibodies**

Multiplex Enzyme linked Immunosorbent assay (ELISA) was performed by coating wells of Nunc Immuo-plates, 96-well, microtiter plates (ThermoScientific, Rochester, NY) with 100-500 ng/well of purified, human recombinant proteins expressing specific neural proteins viz., MAPT, S100B, NSE, GFAP, Tubulin, MAG, NEF, CaMkII α , CaMkII δ and MAP-2. The optimal dilution of the

recombinant protein, sera (serum obtained from GWV) and secondary detection antibody was determined by a checkerboard titration that gave the highest signal to noise ratio to determine the optimum concentration of protein, sera and secondary antibody. In addition, a single lot of antibody directed against these proteins were used to generate quality control standards that gave high and low optical density (high OD = 2.0 to 2.5; low OD = 0.5 to 1.0; and negative OD < 0.2). Briefly, the assay was performed by diluting the recombinant proteins in 15 mM sodium carbonate-35 mM sodium bicarbonate- antigen coating buffer (ACB) pH 9.6. The plates were incubated overnight at 4°C, then washed 3 times (3x) with PBS plus 0.05 % tween₂₀ (PBST). Each well was then blocked with 200 µl of sample buffer (SB) (PBST plus 5 % BSA) and allowed to incubate at room temperature for 3 hrs. Test and control sera were diluted at 1:200 in SB, thorough mixed and 100µl was added to each well, left to incubate/bind at 4°C overnight on a plate shaker. The unbound sera in the wells were washed 3x and secondary antibody (HRP conjugated anti-human IgG) was added at a concentration of 200ng/ml (Jackson laboratory, MA) diluted in SB and incubated at 20-22°C for 1 hour. The plate was then washed 5x and developed with 3,3',5,5'- tetramethylbenzidine, peroxidase substrate (TMB – Invitrogen and incubated in dark until the positive control attained a standard OD. The reaction was stopped using 2N H₂SO₄. Colorimetric development was quantified spectrophotometrically at 450 nm with a Clariostar (BMG plate reader, Germany) using BMG software powered by Matlab programming. The raw data were normalized by subtracting the blank values. Sample to Positive (S/P) ratios were calculated using the following formula: S/P = optical density (OD) of sample - OD of buffer/OD of positive control - OD of buffer. Total IgG levels were determined by quantitating IgG. We also used transferrin as a serum control. All the individual values were normalized to total IgG and transferrin. Final statistical analysis was performed using Matlab and by R.

Presented below are the results of an ELISA assay of autoantibodies against GFAP and MBP



The results are from 3 GWI veterans (p1-p3) and a healthy GW veteran (c1-c3)

- **Western blot assay of Autoantibodies against Neural Proteins**

- a. **Materials and Methods**

Materials

The sources of standard proteins were the same as previously published (Abou-Donia et al., 2013, 2017).

- b. *Case and control Samples*

Serum samples from 20 GWI cases with GWI and 10 controls were tested in this experiment.

- c. *Western Blot Assay*

To screen for the presence of autoantibodies against a battery of proteins in plasma samples, we applied a Western blot approach as previously reported (Abou-Donia et al., 2013, 2017). Each serum sample was analyzed in triplicate. Each protein was loaded as 10-100 ng/lane except for IgG that was loaded as 100 ng/lane. Proteins were denatured and electrophoresed in SDS-PAGE (4% to 20% gradient) purchased from Invitrogen (Life Technology Corporation, ThermoFisher, Grand Island,

NY, USA). One gel was used for each serum sample. The proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech Piscataway, New Jersey). Nonspecific binding sites were blocked with Tris-buffered Saline-Tween (TBST) (40 mM Tris [pH 7.6], 300 mM NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk for 1 h at 22°C. Membranes were incubated with plasma samples at 1:100 dilutions in TBST with 3% non-fat dry milk overnight at 4°C. After five washes in TBST, the membranes were incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Amersham Pharmacia Biotech (Piscataway, New Jersey). The membranes were developed by enhanced chemiluminescence using the manufacturer's (SuperSignal West Femto Maximum Sensitivity Substrate by ThermoFisher Scientific Cat. No. 34096) protocol and a Typhoon 8600 variable mode imager. The signal intensity was quantified using Bio-Rad image analysis software (Hercules, California). All tests were performed with the investigators blinded to participant diagnosis.

Results

- a. Our preliminary results analyzing the current plasma samples are consistent and confirm the results of our preliminary studies using serum samples.
 - b. ELISA assay using the same samples are being analyzed and are showing promising results for future use.
- What opportunities for training and professional development has the project provided?
Professional development. The results of studies that were generated in this project were presented in a national meeting and an international meeting:
 1. The Annual Meeting of the Society of Toxicology (SOT), March 2016, New Orleans: A poster of the results was presented.
 2. The International Neuropsychological Society (INS) annual mid-summer meeting, July 2016, London UK; an oral presentation was given as part of an invited symposium on Gulf War Illness.
 3. The Annual SOT, March 2017, Baltimore, MD: A poster was presented (see Appendix).
 4. Two additional posters will be presented at SOT meeting in San Antonio, TX in March 2018.
 - How were the results disseminated to communities of interest?
 - Our results were discussed with many interested scientists during both the SOT and INS meetings.
 - A symposium on Gulf War Illness took place in London during the International Neuropsychological Society mid-year meeting last July, during which results from this project and several other related projects were presented and stirred intense interests.
 - We published our first manuscript of CNS autoantibodies in the Journal Neurotoxicology and Teratology (see attached and below) we were also publicized in a local media story at <https://www.bu.edu/sph/2017/03/29/identifying-biomarkers-of-gulf-war-illness>
 Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR, Sullivan K. Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. *Neurotoxicol Teratol.* 2017 May; 61:36-46. PMID: 28286177.
 - What do you plan to do during the next reporting period to accomplish the goals?
 - We plan to attend appropriate National and International meetings to present our results from this project.
 - Annual Meeting of the Society of Toxicology, March 2018, San Antonio, TX. Two abstracts have been submitted for poster presentations.
 - We plan to submit other manuscripts of preliminary results from the case-control and exposed vs non-exposed groups and the new ELISA method during the next reporting period.
4. Impact
 - What was the impact on the development of the principal discipline(s) of the project?

- Our studies have focused on the development of sensitive, specific, reproducible, and non-invasive blood biomarkers of GWI. Identifying objective biomarkers of GWI helps the veteran and the treating clinicians who must now rely on self-report of symptoms as the primary diagnostic marker. The advantage of a blood-based biomarker is that it can diagnose GWI with greater accuracy and with only a few drops of blood. Our initiative to validate both serum and plasma for these potentially diagnostic autoantibodies will make diagnosing GWI and validating it with other stored blood samples from GWI even easier because it will not be limited by just one type of blood product.
- The results of the study can be applied immediately to treatment development strategies for the veterans of the Gulf War. Based on the CNS autoantibodies we ultimately find, this will provide the opportunity to develop drugs that treat neuronal injury in those specific pathways (neuronal, glial etc.); such treatment could be directly applicable to Gulf War veterans in the short-term.
- Our first publication provides the CNS autoantibodies that we will attempt to validate in our larger study sample and target for treatment development planning.
- What was the impact on other disciplines?
 - A major advantage of our peripheral marker is that it is specific for neural injury irrespective of the cause, thus it can be applied to diagnose or confirm diagnosis of other neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. However, the *pattern* of the specific markers elevated can be different with different disease states and thus can be useful diagnostically once validated in different disorders. For example, we have recently found different patterns of CNS autoantibodies in CFS, IBS and GWI that we will report further in a publication.
 - Blood-based biomarkers of GWI provide an effective way to enhance its management
 - It can be used as a diagnostic and prognostic tool with the ability to provide information about rate of disease progression.
 - It would help in identification of novel and effective treatments for multiple disorders and environmental exposure groups (i.e. pesticides, nerve agents).
 - It could be used for monitoring therapeutic efficacy for the benefit of patients and caregivers.
 - It could be used to follow-up treatment plans of the patient
 - It could provide a cost-savings potential for recruitment into clinical trials.
- What was the impact on technology transfer?
 - The biomarker that we developed is cost effective, will be made available to clinical laboratories and recommended for routine screening procedure.
- What was the impact on society beyond science and technology?
 - Our peripheral biomarker should improve the quality of life for the veterans of the GW who have GW illness because:
 - Upon their returning from the GW theater operations in 1991, their subjective complaints could not be diagnosed and they were told that their complaints were "all in their heads". Our biomarker should confirm brain injury that is consistent with their complaints. Such consequence should give them a peace of mind.

Our biomarker should lead studies to develop treatment for brain injury that would provide improvement of their clinical condition.

The hallmark of Gulf War Illness (GWI) is neuroinflammation, neural cell death in specific regions of the brain and possible progressive neurodegeneration. A challenging aspect of GWI is that it has been difficult to diagnose with objective biomarkers because organophosphate pesticides and nerve

agents do not stay in the body and CNS the same way that other exposures do (i.e. Agent Orange, depleted uranium, lead, and mercury). Therefore, researchers have had to develop markers of damage from these chronic exposures rather than markers of the exposure or their bi-products. If successful, this work will impact neurotoxicant exposed individuals including agricultural workers, pesticide applicators and nerve gas exposed groups by providing objective inexpensive markers of chronic damage relating to these exposures that can be conducted virtually anywhere that a simple blood draw can be obtained and analyzed. Other current diagnostic practices including neuroimaging techniques, behavioral history assessments, and neuropsychiatric tests have drawbacks of not being practical or available in other parts of the world but a simple blood test could provide objective diagnostic markers in the most cost-effective way.

5. Changes for approach and reasons for change

- Changes: serum to plasma samples

Our original studies in determining autoantibodies in blood, used serum samples from GWI cases and symptomatic controls. However, it was determined that our co-investigators had plasma available than serum. Therefore, we carried out experiments to establish the validity of our assay using plasma, as stated above under ‘Accomplishments’, we showed that the results from plasma samples were identical to those of serum samples. This was a big accomplishment that either plasma or serum can be used for these analyses because all other major studies of GWI with either serum or plasma samples could potentially validate our findings with their own samples.

- Problems:

None

- Actual or anticipated problems or delays and actions or plans to resolve the techniques
 - We have been slightly delayed in getting CSF samples for analysis because the GWIC is still obtaining them but there should not be a long delay before they are ready for sharing.
- Changes that had significant impact on expenditures
 - None
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
 - Significant changes in use or care of human subjects: None
 - Significant changes in use or care of vertebrate animals: None
 - Significant changes in use of biohazards, and/or select agents: None

6. Products

- **Publications, conference papers, and presentations**

- **Journal Publications**

- Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR, Sullivan K. 2017. Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. *Neurotoxicol Teratol*.61:36-46. See attachment 1.

- **Books or other non-periodicals, one-time publications**

- None

- **Other publications, conference papers, and presentations**

- A poster was presented during the annual Society of Toxicology Meeting at Baltimore, MD entitled:

“A Pilot Study of Novel Brain Neurodegenerative Biomarkers in Veterans with Gulf War Illness: M. B. Abou-Donia¹, K. Sullivan², L. A. Conboy³, E. Kokkotou⁴, and E. M. El-Masry⁵ ¹Duke University Medical Center, Durham, NC, ² Boston University School of Public Health, Boston, MA, ³Harvard Medical School, Boston, MA, ⁴ Harvard Medical School. Boston, MA ; ⁵ Zagazig University, Zagazig, Egypt”

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○ **Other products**

● **Website(s) or other Internet site(s)**

● **None**

● **Technologies or techniques**

● **None**

● **Other products**

● **None**

7. Participants & other Participating Organizations

Site 1: Boston University School of Public Health 715 Albany Street, T4W Boston, MA 02118 Initiating PI: Dr. Kimberly Sullivan Co-I: Dr. Joseph Massaro Co-I: Dr. Maxine Krengel Tasks 1-6	○ Duke University Medical Center Durham, North Carolina 27710 Partnering PI: Dr. Mohamed Abou Donia Co-I: Dr. Cameron R. ‘Dale’ Bass Tasks 2,3,5,6
Site 3: National Institutes of Health, NICHD Bldg. 35, Room 2A211, MSC 3713 35 Lincoln Drive Bethesda, MD 20892 Site PI: Dr. R. Douglas Fields Co-I: Dr. Dipankar Dutta Tasks 2-6	<u>Blood Serum and CSF Biorepository Sites</u> Site 4: NOVA Southeastern University Ft. Lauderdale, FL Co-I: Dr. Nancy Klimas Tasks 1,2, 5, 6 Site 5: Beth Israel Deaconess Medical Ctr. Boston, MA 02118 Consultant: Dr. Efi Kokkotou Consultant: Dr. Lisa Conboy Tasks 1,2, 5,6

Study Sites Responsibilities

Site 1: Dr. Sullivan and her BUSPH team will be responsible for providing the serum blood and cerebrospinal fluid samples from GWIC study participants who have agreed to share their specimens with the GWIC biorepository to be used in future studies including the proposed study. Specifically, she will oversee the recruitment and blood draws/lumbar punctures of study participants from the GWIC study and the processing of serum and CSF samples that will be shared for the proposed study. Dr. Sullivan will also assist with the experimental design, data analysis, interpretation and presentation of study results in collaboration with Dr. Abou Donia and the other study investigators. **Tasks 1-6**

Site 2: Dr. Abou-Donia will be responsible for receiving the serum and plasma samples from all sites and performing autoantibody analyses using western blot/ELISA analyses for 450 serum samples (250 GWI, 200 controls). He will also assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2, 3, 5, 6**

Site 3: Dr. Fields will be responsible for receiving the serum and CSF samples from all study sites and performing ELISA assays for 450 serum/plasma samples and 50 CSF samples. He will assist with the experimental design, interpretation of data, report and manuscript writing and presentation of results at scientific meetings. **Tasks 2-6**

Sites 4 and 5: Drs. Klimas, Conboy and Kokkoutu will provide serum samples from their respective biorepositories for study analyses, assist with interpretation of data, report and manuscript writing.

8. Special Reporting Requirements

None

APPENDICES

Appendix 1: Please see attached manuscript for Abou-Donia et al., 2017, entitled: “Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR, Sullivan K. (2017). Neurotoxicol Teratol.;61:36-46”.

Appendix 2:

Abstract: Annual Society of Toxicology Meeting, Baltimore, MD, March, 2017

Neural Autoantibodies in Veterans with Gulf War Illness. M.B. Abou-Donia¹, K. Sullivan² L. Conboy³, E. Kokkotou.⁴

¹Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 2 Boston University School of Public Health, Boston, MA; ³Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; ⁴New England School of Acupuncture, Harvard Medical School, Boston, MA.

A number of studies have linked exposures of chemical and biological toxins to increased risk of autoimmunity and elevated levels of antibodies to neural antigens. Currently, autoimmune diseases affect 5-7% of the world's population that are usually accompanied by circulating autoantibodies. Neural autoantibodies are also present in approximately 2-3% worldwide, but have not been involved in brain pathology. This current study measured levels of 8 types of neural autoantibodies in a group of 20 Gulf War patients with Gulf War Illness (GWI). The patients were exposed to a variety of toxicants including insecticides, insect repellent, pyridostigmine bromide, a prophylaxis for nerve agents and the nerve agent sarin. The results indicate that neural markers were significantly elevated in peripheral blood samples of GWI cases. Of interest, the highest fold increase (8 times that of controls) was found in GFAP, a known marker of astrocyte activation. Other markers of axonal transport damage were also significantly increased in GWI cases including MAP, Tau, tubulin and NFP. Myelin basic protein, a marker of oligodendrocytes, was also increased. These results strongly corroborate with the GWI hypotheses that neuroinflammation in GWI potentially results in white matter and axonal transport damage. Autoantibodies directed against self-antigens can also cause local activation of complement. *B cells play a key role in autoimmunity, B cell lineage may contribute to the development either as antigen presenting cells or cytokine secreting cells or autoantibody producing cells. Immunotherapies that deplete B cells may be an effective strategy to combat autoantibodies.* (Supported in part by DOD Contract No. W81XWH-15-1-0641).

Appendix 3:

Abstract: Annual Society of Toxicology Meeting, San Antonio, TX, March 2018

Biomarkers for Chronic Fatigue Syndrome (CFS) and Irritable Bowel Syndrome (IBS) compared to Gulf War Illness (GWI) in Gulf War Veterans.

M. B. Abou-Donia¹, M. V. Brahmajothi¹, L. A. Conboy², E. Kokkotou³, E. Jacobson⁴, N. Klimas⁵, and K. Sullivan⁶.

¹Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC; ²Medicine, Harvard Medical School, Boston, MA; ³Gastroenterology, Harvard Medical School, Boston, MA; ⁴Biostatistics, Harvard Medical School, Boston, NC; ⁵Neuro-Immune Medicine, NOVA Southeastern University, Davie, FL; and ⁶Environmental Health, Boston University, Boston, MA.

Approximately one third of the American military personnel who served in the Gulf War from August 1990 to June 1991 experienced chronic symptoms of GWI. Other Gulf War Veterans' illnesses included CFS and IBS. CFS is characterized by debilitating fatigue, unrefreshing sleep, neuropsychological decrements and chronic pain. IBS is characterized by recurrent abdominal pain and bowel difficulties. In

the present study, we used our newly developed biomarkers using western blot assay to screen serum for the presence of autoantibodies (AA) against the following neural proteins: neurofilament triplet proteins (NFP), tubulin, microtubule associated tau proteins (tau), microtubule associated protein-2 (MAP-2), myelin basic protein (MBP), myelin associated glycoprotein (MAG), calcium-calmodulin kinase II (CaM-KII) and glial S100B protein. Serum reactivity was measured as arbitrary chemiluminescence units. The study included 50 CFS, 50 IBS, 68 GWI and 26 non-veteran asymptomatic served as controls. None of the patients showed any significant increase in AA against S100B. Patients with CFS showed statistically significant increase in AA only against four proteins over controls, presented in descending order: MAP-2 (2.90) > GFAP (2.88) > Tubulin (2.05) > CaMkII (1.51) > MBP (1.48) > SNCA (1.42) > NFP (1.21) > MAG (1.176) > Tau (1.04). IBS patients exhibited the following pattern: MAG (3.20) > MBP (1.47) > Tau (1.39) > SNCL (1.39). GWI patients showed the following pattern: MAP-2 (4.81) > MBP (3.93) > NFP (3.63) > Tubulin (3.48) > Tau (3.17) > MAG (3.14) > SNCL (3.03) > GFAP (2.68) > CaMKII (2.48). These results showed that patients with CFS and IBS have less levels of AA against fewer neural proteins, indicating that the levels of AA against neural proteins in these patients are below the threshold levels that accompany brain injury in contrast to patients with GWI and in agreement with absence of neurological symptom complaints in these patients. (Supported in part by DOD Contract No. W81XWH-15-1-0641).

Appendix 4:

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Quantitative measurement of Neural Autoantibodies to Map-2, Tau, Tubulin and GFAP proteins by ELISA in the sera of patients with Gulf War Illness (GWI).

M.V. Brahmajothi¹, L.A. Conboy², E. Kokkotou³, K. Sullivan⁴, N. Klimas⁵, and M.B. Abou-donia¹.

¹Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC; ²Public Health, Harvard Medical School, Boston, MA; ³Medicine, Harvard Medical School, Boston, MA; ⁴Environmental Health, Boston University, Boston, MA; and ⁵Immunology, NOVA Southeastern, Davie, FL.

For Gulf War Veterans (GWV), the effects of war continued long after they returned home. In addition to the psychological ramifications, veterans and civilian workers showed exacerbated effects of medically unexplained chronic multi-system disorders. The causality of illness may be due to the compounds they were exposed to, which inhibit acetylcholine (AChE) or modulate the pharmacokinetics of substances that control the metabolic activation or breakdown of AChE inhibitors. Such compounds include chlorpyrifos, sarin, sulfur sarin, sulfur mustard, pyridostigmine bromide, DEET, opiates as therapeutics and the enzymes responsible for drug metabolism such as cytochrome P450 reductases, liver microsomal oxidases, etc. Inaccessibility of the nervous system has impeded the evaluation of cellular and molecular changes that result in neurodegeneration. Discharges of neural proteins during this process can induce autoimmune response that can be measured in the serum. When we screened for novel nervous system biomarkers in the sera of GWVs from our pilot study, we found 2 to 9-fold increase of autoantibodies to the neuronal specific proteins. We have now developed ELISA to determine and quantitate serum autoantibodies against microtubule associated proteins (MAP-2), microtubule associated protein tau (Tau), tubulin and glial fibrillary acidic protein (GFAP). This method quantitatively distinguishes IgG levels of the autoantibody titers at 0.1 microgram level of the specific neuronal proteins. Determination of specificity was achieved by absorption studies to estimate the threshold level. We performed dose response to determine the optimum concentration of each protein. (Supported in part by DOD Contract No. W81XWH-15-1-0641).



Full length article

Screening for novel central nervous system biomarkers in veterans with Gulf War Illness

Mohamed B. Abou-Donia ^{a,*}, Lisa A. Conboy ^b, Efi Kokkotou ^c, Eric Jacobson ^{dj}, Eman M. Elmasry ^e,
 Passent Elkafrawy ^f, Megan Neely ^g, Cameron R. 'Dale' Bass ^h, Kimberly Sullivan ^h

^a Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, United States

^b Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States

^c Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States

^d Department of Global Health and Social Development, Harvard Medical School, United States

^e Department of Microbiology, Zagazig University, Zagazig, Egypt

^f Department of Math and Computer Science, Menoufia University, Shebin El-Khayma, Egypt

^g Department of Biostatistics & Bioinformatics, Duke University Medical Center, United States

^h Biomedical Engineering Department, Duke University, United States

ⁱ Department of Environmental Health, Boston University School of Public Health, Boston, MA, United States

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ABSTRACT

Gulf War illness (GWI) is primarily diagnosed by symptom report; objective biomarkers are needed that distinguish those with GWI. Prior chemical exposures during deployment have been associated in epidemiologic studies with altered central nervous system functioning in veterans with GWI. Previous studies from our group have demonstrated the presence of autoantibodies to essential neuronal and glial proteins in patients with brain injury and autoantibodies have been identified as candidate objective markers that may distinguish GWI. Here, we screened the serum of 20 veterans with GWI and 10 non-veteran symptomatic (low back pain) controls for the presence of such autoantibodies using Western blot analysis against the following proteins: neurofilament triplet proteins (NFP), tubulin, microtubule associated tau proteins (Tau), microtubule associated protein-2 (MAP-2), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP), calcium-calmodulin kinase II (CaMKII) and glial S-100B protein. Serum reactivity was measured as arbitrary chemiluminescence units. As a group, veterans with GWI had statistically significantly higher levels of autoantibody reactivity in all proteins examined except S-100B. Fold increase of the cases relative to controls in descending order were: CaMKII 9.27, GFAP 6.60, Tau 4.83, Tubulin 4.41, MAG 3.60, MBP 2.50, NFP 2.45, MAP-2 2.30, S-100B 1.03. These results confirm the continuing presence of neuronal injury/gliosis in these veterans and are in agreement with the recent reports indicating that 25 years after the war, the health of veterans with GWI is not improving and may be getting worse. Such serum autoantibodies may prove useful as biomarkers of GWI, upon validation of the findings using larger cohorts.

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1. Introduction

Approximately one third of the 697,000 US military personnel who served in the Gulf War (GW) from August 1990 to June 1991, have reported persistent symptoms for many years after the war (RAC, 2008; IOM, 2012; RAC, 2016; White et al., 2016). This complex of symptoms, known as Gulf War Illness (GWI), include memory and attention problems, profound fatigue, chronic muscle and joint pain, severe headaches,

persistent diarrhea, respiratory difficulties and skin rashes. GWI is primarily diagnosed by symptom report and no validated objective diagnostic biomarkers currently exist that fully segregate cases from controls. This study was designed to identify objective central nervous system (CNS) biomarkers of GWI using clues from prior clinical studies with GW veterans and from animal studies that modeled chemical exposures experienced by GW veterans.

Clinical studies have reported impaired cognitive functioning and reduced MRI volume and altered white matter microstructural integrity in organophosphate (OP) pesticide, sarin nerve agent and pyridostigmine bromide (PB) anti-nerve gas pill-exposed GW veteran cohorts (White et al., 2016; Sullivan et al., 2013; Chao et al., 2010;

* Corresponding author at: Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, United States.
 E-mail address: donia@duke.edu (M.B. Abou-Donia).

Heaton et al., 2007; Proctor et al., 2006; Sullivan et al., 2003). Animal studies demonstrated that exposure to higher doses of the prophylaxis pill pyridostigmine bromide (PB), the insect repellent, DEET, and the insecticide permethrin and/or chlorpyrifos led to significant brain damage in animal models of GWI (Abou-Donia et al., 1996a,b). Further studies using 60 days of subchronic dermal exposure to DEET and permethrin, alone or in combination, at dose levels approximately equivalent to the exposures that occurred during the Gulf War in a rat-model of GWI, caused the following: (1) a diffuse neuronal cell death in the motor cortex, the different subfields of the hippocampal formation, and the Purkinje cell layer of the cerebellum, accompanied by sensorimotor deficits; (2) significant reduction of MAP-2-positive immunoreactive structures indicating atypical expression of MAP-2 in dendrites of surviving neurons, within the cerebral cortex and the hippocampus that was characterized by a beaded, disrupted, or wavy appearance; (3) a significant upregulation of GFAP-positive expression in structures in the CA3 subfield of the hippocampus, the motor cortex and the dentate gyrus (Abdel-Rahman et al., 2001, 2002a,b, 2004a,b; Abou-Donia et al., 2000, 2001, 2002, 2004; Terry et al., 2003). Similar results were exhibited in animals treated with sarin alone or accompanied by cited-above chemicals, with and without stress (Abdel-Rahman et al., 2004a).

The cytoarchitecture of the CNS is maintained by a complex cellular milieu that involves neuronal and glial cells that must maintain proper communication in order to function properly (Abou-Donia and Lapadula, 1990; McMurray, 2000). CaMKII phosphorylates cytoskeletal proteins, such as MAP-2, tau and tubulin. CaMKII accounts for 12% of all proteins in the brain. CaMKII has the ability to coordinate and transduce upstream Ca and reactive oxygen species (ROS) signals into physiological and pathophysiological downstream responses in the nervous system and cardiovascular biology and disease (Abou-Donia, 1995; Erickson et al., 2011). Tubulin, the major component of microtubules, is responsible for axonal migration and longitudinal growth and is involved in axonal transport. Although tubulin is present in virtually all eukaryotic cells, the most abundant source is the vertebrate brain, where it consists of approximately 10–20% of its total soluble protein (McMurray, 2000). Microtubule-Associated Protein-2 (MAP-2) is found in dendritic compartments of neurons. A loss of MAP-2, is a reliable indication of irreversible neuropathology and is a sensitive marker of seizure-related brain damage (Balloogh et al., 1995). Tau Protein, a normal axonal protein, is involved in stabilization and assembly of axonal microtubules. Levels of tau proteins are elevated in the cerebrospinal fluid (CSF) and serum following TBI (Liliang et al., 2011) and has been used for diagnosis of Alzheimer's disease. Myelin basic protein (MBP) is an abundant myelin membrane proteolipid produced by oligodendroglia in the CNS and Schwann cells in PNS and may confirm the clinical assessment of neurodegenerative disorders such as multiple sclerosis and stroke (Jauch et al., 2006). Myelin Associated Glycoprotein (MAG) is selectively localized in periaxonal Schwann cell and oligodendroglial membranes of myelin sheaths, suggesting that it functions in glia-axon interactions in both the PNS and CNS (Schachner and Bartsch, 2000). Glial fibrillary acidic protein (GFAP) is expressed almost exclusively in astrocytes, where it is induced by neural injury and released upon disintegration of the astrocyte cytoskeleton (Rempe and Nedergaard, 2010). GFAP plays an essential role in maintaining shape and motility of astrocytic processes and contribute to white matter architecture, myelination and blood brain barrier (BBB) integrity (O'Callaghan et al., 2015). After traumatic brain injury (TBI), GFAP's serum concentration peaks at 2–6 h and has a half-life of <2 days (Diaz-Arastia et al., 2014). S-100B exerts both detrimental and neurotrophic effects, depending on its concentration in brain tissues (Adami et al., 2001). After release, S-100B acts as a trophic factor for serotonergic neurons, and plays a role in axonal growth and synaptogenesis during development. Thus, traumatic acute injury results in great destruction of astrocytes leading to massive release (50 to 100 fold) of S-100B into plasma, whereas S-100B levels in psychiatric disorders were

only about 3 times higher in patients compared to controls (Uda et al., 1998; Arolt et al., 2003), correlating well with its neuroprotective action. Specifically, S-100B stabilizes tau and MAP-2. Its half-life in the serum is 2 h (Zurek and Fedora, 2012).

A recent study of airline pilots and other flight crew members chronically exposed to organophosphates through combustion of engine oil and hydraulic fluid that contain organophosphate esters resulted in symptoms similar to those reported by GW veterans (fatigue, headaches, confusion and memory problems). Interestingly, these crew members showed significantly elevated numbers of autoantibodies in their blood serum of CNS damage markers including those associated with axonal transport (microtubule associated protein-2 (MAP-2), tubulin, neurofilament triplet proteins (NFP) and microtubule associated protein-tau (tau protein)) and those exclusively associated with CNS glial activation and neuroinflammation (myelin basic protein (MBP), and glial fibrillary acidic protein (GFAP) (Abou-Donia et al., 2013). A follow-up histopathology autopsy study was performed on a deceased pilot with organophosphate exposure that confirmed CNS damage and demyelination (Abou-Donia et al., 2014). Specifically, the histopathology results showed axonal degeneration and demyelination and the post-mortem and pathological examination of the nervous system confirmed the autoantibody biomarker results.

Recent studies with GW veterans have shown persistent signs and symptoms characteristic of CNS injury including brain imaging and cognitive studies (White et al., 2016; Chao et al., 2010, 2011, 2014, 2016; Heaton et al., 2007; Sullivan et al., 2003). There are, however, no validated objective diagnostic tests to identify acute or chronic sequelae of brain injury in this veteran group. Diagnosis of brain injury using cranial computed tomography (CT) scan and magnetic resonance imaging (MRI) techniques such as diffusion tensor imaging (DTI), have not been able to clinically diagnose veterans with GWI because there have been no proven cutoff values for volumetric or other imaging parameters that have been able to provide the required near 100% accuracy in terms of sensitivity/specificity at the individual level to distinguish cases from controls needed for a diagnostic test. Imaging studies have been able to show differences and altered CNS functioning between veterans with GWI and healthy controls but have not yet been able to identify the groups diagnostically because of the significant overlap between the groups (Chao et al., 2010, 2011, 2014, 2016; Heaton et al., 2007). Hence, it is important to develop clinically available, simple and inexpensive biomarkers for detection of neuronal and glial injury essential in the diagnosis and understanding of the temporal progression of CNS damage in GWI. Recently, serum biomarkers such as cytoskeletal proteins, resulting from axonal degeneration, have been used in diagnosing brain injury (particularly traumatic brain injury). The use of these biomarkers is usually measured in serum shortly after brain injury, because they have short half-lives (Zurek and Fedora, 2011; Diaz-Arastia et al., 2014).

However, many years have elapsed since the time that GW veterans returned from deployment and became ill therefore, this particular approach cannot apply to GWI. Based on results from both chronic and acute injury, we used our novel autoantibody biomarker panel described above for brain injury to test for the indication of CNS damage in veterans with chronic GWI (Abou-Donia et al., 2013, 2014). One prior study compared autoantibodies of myelin basic protein (MBP) and striated muscle antibodies in GW veterans and reported higher MBP and muscle antibodies in veterans with GWI (Vojdani and Thrasher, 2004). Autoantibodies have previously been recognized as potential objective biomarkers of GWI (Golomb, 2012). Therefore, we hypothesized that chemical exposure to pesticides, anti-nerve gas pills and/or sarin nerve gas during deployment in veterans with GWI caused an excitotoxic cascade (through potential glutamatergic, oxidative stress and proinflammatory cytokine signaling) resulting in neurodegeneration and apoptotic loss of brain cells, leading to blood brain barrier leakage of specific neuronal and glial proteins into circulation, with subsequent formation of autoantibodies (AB) against these

proteins (Abou-Donia et al., 2013; Banks and Lein, 2012; Golomb, 2008; Terry, 2012; Binukumar and Gill, 2010; Soltaninejad and Abdollahi, 2009). In this study, we determined circulating IgG- class autoantibodies in serum from 20 GWI cases and 10 symptomatic (low back pain) controls against the following 9 brain proteins: neurofilament triplet proteins (NFP), tubulin, microtubule associated protein-tau (tau proteins), microtubule associated protein-2 (MAP-2), calcium/calmodulin Kinase II (CaMKII), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP) and S-100B.

2. Materials and methods

2.1. Materials

The sources of proteins were: NFP (bovine spinal cord), tau protein (human), MAP-2 (bovine serum), tubulin (bovine brain), and MBP (human brain), from Sigma-Aldrich (Saint Louis, Missouri); CaMKII (Human) recombinant Protein and MAG recombinant Protein from Novus Biologicals, Littleton, CO, GFAP (human) from Biotrend Chemikalien GmbH, (Cologne, Germany) and S-100B (human brain) from American Qualex International, Inc. (San Clemente, California). Horseradish peroxidase-conjugated goat anti-human IgG, and enhanced chemiluminescence reagent were obtained from Amersham Pharmacia Biotech (Piscataway, New Jersey). SDS gels, 2–20% gradient (8 × 8), and tris-glycine 15 mM were obtained from Invitrogen (Carlsbad, California). All other materials were purchased from Amersham.

2.2. Ethics statement

Approval for the use of stored blood samples for this study was obtained from the Duke University Medical Center Institutional Review Board.

2.3. Case and control samples

Serum samples from 20 GWI cases with GWI and 10 non-veteran symptomatic controls with lower back pain were tested in this pilot study. GWI veteran serum samples were collected from a study of acupuncture treatment in veterans with GWI from 2010 to 2012 (Conboy et al., 2012). Control serum samples were derived from a separate study of non-veteran patients with chronic lower back pain who served as 'symptomatic low back pain' controls from 2011 to 2013 (Jacobson et al., 2015). Veterans with GWI will be referred to as 'cases' and low-back pain symptomatic controls will be referred to as 'controls'.

2.4. Description of the patient cohorts

2.4.1. GWI-case cohort

"The Effectiveness of Acupuncture in the Treatment of Gulf War Illness" P1: Conboy, (8/21/2010–12/26/2012) N = 104; Study Site: New England School of Acupuncture (NESA). Cases were recruited through the Defense Manpower Data Base (DMDC) personnel listings and advertisements. Cases were screened for GWI symptoms and were required to meet the CDC diagnostic criteria for chronic multi-symptom illness (CMI) in order for inclusion in the parent study and in the current study (Conboy et al., 2012; Fukuda et al., 1998). Inclusion in the current study also required that veterans were deployed to the 1990–1991 Gulf War. CMI is characterized by one or more symptoms of at least 6 months duration from at least two of three symptom categories: 1) fatigue; 2) mood-cognition; 3) musculoskeletal pain.

Symptoms were not necessarily required to have started during or after the Gulf War deployment. Exclusionary criteria included that the veteran was 1) currently enrolled in another clinical trial 2) Had another disease that likely could account for the symptoms, as determined by the Medical Monitor 3) Severe psychiatric illness (in the last 2 years psychiatric hospitalization, suicidal attempt, alcohol or substance

abuse, use of antipsychotic medication) 4) Unable to complete the protocol based on the evaluation of the Medical Monitor.

2.4.2. cLBP-cohort

"Structural Integration for chronic low back pain" P1: Jacobson (3/4/2011–6/21/2013) N = 46. Study Site: Spaulding Rehabilitation Hospital (SRH). In this cohort, 46 outpatients from the Boston area with chronic nonspecific low back pain were randomized to parallel 20-week long treatment groups of structural integration (SI) plus outpatient rehabilitation (OR) versus OR alone. The details of the study are described in a recent publication (Jacobson et al., 2015). Inclusion criteria for the parent study included: (i) Men and women aged 18–65, (ii) cLBP of ≥6 months duration, not attributed to infection, neoplasm, severe radiculopathy (as indicated by frequent severe pain radiating down a leg), fracture, or inflammatory rheumatic process, (iii) bothersomeness of back pain self-rated on average over the preceding 6 months ≥3 on an 11-point ordinal scale (0 = none, 10 = worst imaginable), (iv) prior arrangement to enter a course of outpatient physical therapy for low back pain at a Boston area rehabilitation clinic, (v) English language fluency and mental capacity sufficient to provide informed consent and participate in the study. Exclusion Criteria for the study included: (i) Impaired hearing, speech, vision, and mobility sufficient to interfere with participation in the study, (ii) current or anticipated receipt of payments from Worker's Compensation or other insurance for disability attributed to low back pain, (iii) prior treatment with any SI therapy, (iv) plans to initiate additional treatment for back pain during the period of the study other than outpatient rehabilitation care, particularly massage or other manual therapies (e.g., chiropractic or osteopathic manipulation), (v) exclusions for safety: unresolved musculoskeletal pathology of the lower limbs, current pregnancy, any implanted medical device, osteoporosis, any hypercoagulation condition, eczema, skin infection, deep vein thrombosis, burns or other acute trauma including unhealed bone fractures or open wounds, psoriasis, psychiatric illness not well controlled, or current episode of exacerbated major depressive disorder.

2.5. Collection and storage of samples

Samples from the GWI-cohort and the cLBP-cohort were all collected from the Boston area at the same time period at two different sites from 2010 to 2013. All sites followed exactly the same protocol for venipuncture, blood handling, serum separation, aliquoting and storage at −80 °C. The same phlebotomy and sample protocol was distributed in writing to all sites. All samples analyzed were baseline blood samples collected pre-intervention therapy. Samples used for this study have not been previously thawed and are free of hemolysis by visual inspection (Tuck et al., 2009).

2.6. Participant demographics

The participant demographics indicate that a total of 20 veterans with GWI, 18 males and 2 females, compared to 6 females out of 10 cLBP controls participated in the study. The age of the GWI cases ranged from 38 to 61 (mean ± SD 46.0 ± 6.8) compared to 25 to 64 (mean ± SD 50 ± 11.4) years for controls; all study participants were white (Table 1). Seventy percent of veterans with GWI reported taking PB

Table 1
Study participant demographics.^a

Demographics	Cases	Controls
Age (mean ± SD)	46 (6.4)	50 (11.4)
Gender (% female) ^b	10	60
Race (% Caucasian)	100	100

Age range of Cases = 38–61 years and Controls = 25–64 years in 2010–2013 when the blood was collected.

^a A total of 20 cases and 10 controls participated in the study.

^b Cases were significantly different from controls for gender $p < 0.05$ but not for age.

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pills during the war ($n = 14$). The groups differed with respect to gender ($X^2 = 8.5$; $p < 0.05$) with significantly more women in the control group but did not differ with respect to age (t -value = -1.3 ; $p > 0.05$).

2.7. Western blot assay

To screen for the presence of autoantibodies against a battery of proteins, we applied a Western blot approach as previously reported (Abou-Donia et al., 2013). Each serum sample was analyzed in triplicate. Each protein was loaded as 10 ng/lane except for IgG that was loaded as 100 ng/lane. Proteins were denatured and electrophoresed in SDS-PAGE (4% to 20% gradient) purchased from Invitrogen (Carlsbad, CA). One gel was used for each serum sample. The proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech Piscataway, New Jersey). Nonspecific binding sites were blocked with Tris-buffered Saline-Tween (TBST) (40 mM Tris [pH 7.6], 300 mM NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk for 1 h at 22 °C. Membranes were incubated with serum samples at 1:100 dilutions in TBST with 3% non-fat dry milk overnight at 4 °C. After five washes in TBST, the membranes were incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Amersham Pharmacia Biotech (Piscataway, New Jersey). The dot blots were probed with anti-human IgG (H + L) HRP conjugate antibody (Cat. No. 31410, Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) for 1 h at RT, incubated with ECL reagent (Cat. No. 34096). The membranes were developed by enhanced chemiluminescence using the manufacturer's (Amersham Pharmacia Biotech) protocol and a Typhoon 8600 variable mode imager. The signal intensity was quantified using Bio-Rad image analysis software (Hercules, California). All tests were performed with the investigators blinded to participant diagnosis.

2.8. Specificity of serum autoantibodies

Previously we checked the specificity of the serum autoantibody by performing peptide/antigen competition assay, in which the serum was spiked with the target protein or peptide (Abou-Donia et al., 2013). The serum from random healthy controls was mixed with or without tau, MAP or MBP. The serum/protein mix was centrifuged at 15,000 rpm to pellet any immune complexes. The supernatants were then carefully removed and used in Western blotting.

2.9. Calculations

The mean value of the optical density measurement from the triplicate testing was used for each serum sample tested and normalized by total IgG. Thus, the results are expressed as mean values of triplicate assays of optical density arbitrary units normalized to total serum IgG.

2.10. Power analysis

A total of 20 GWI cases were available for testing in this convenience sample. Effect size calculations were based on two-sample t -test assuming a common standard deviation between groups. The power analysis assumes that cases and controls are not matched. In a t -test of difference between two independent means, selecting power of 80%, 2-sided α 0.05, and size of 20 vs 10, the study was powered to detect an effect only if at least 1.12 SD.

2.11. Statistics

Grouped data are reported as mean \pm SD. The values from cases were compared to the control group using t -tests and Pearson correlation analyses (SigmaStat, Systat Software) and p -values were

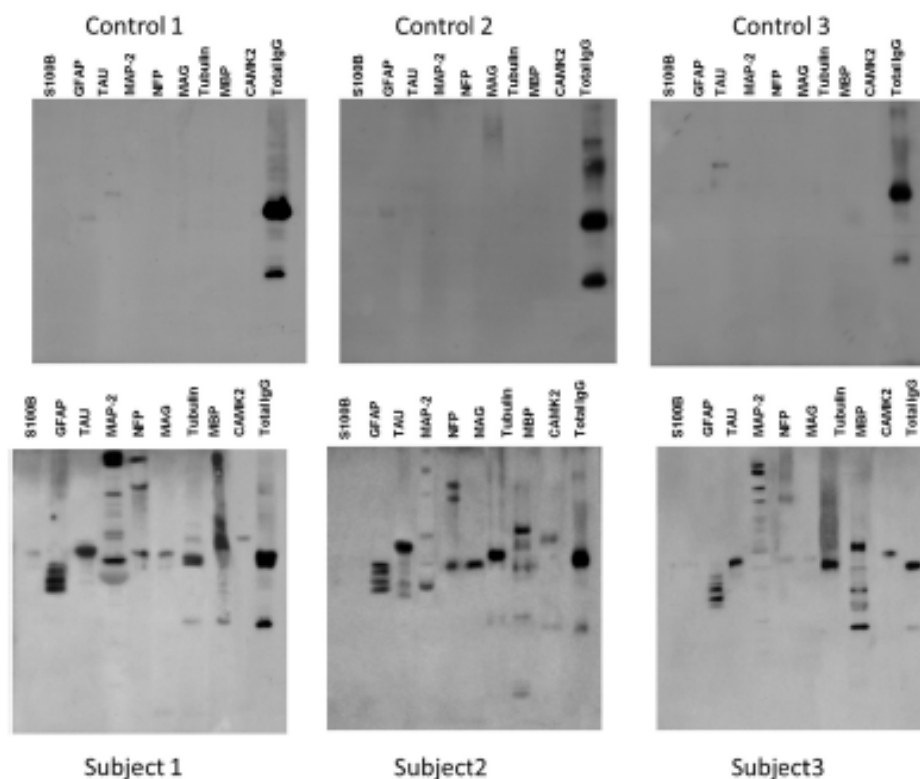


Fig. 1. A representative sample of Western blot gels from three cases showing that the majority of GWI serum reacted intensely to neural proteins, while most control serum showed a weak or no reaction.

Table 2
Chemicals, environmental and other exposures of cases during the Gulf War.^a

Chemical exposures			Environmental and other exposures		
	Exposed	%		Exposed	%
Pyridostigmine bromide (PB)	14	70	Khamisiyah notification letter	8	40
Organophosphorus pesticides (OP)	7	35	Contaminated food/water	18	90
Carbamates	7	35	Vaccines	18	90
Pyrethroids	4	20	Malaria	12	60
DEET	11	55	Sand	18	90
Sarin	9	45	Tent heater	11	55
Depleted uranium (DU)	6	30	Jet fuel	14	70
Solvents	10	50	Oil fires	18	90

^a A total of 20 veterans with GWI participated in the study.

calculated. Pairwise correlations among the nine biomarkers were assessed. A 2-sided *p* value < 0.05 was considered significant. Due to the exploratory nature of this pilot study, analyses were not adjusted for multiple comparisons.

3. Results

As previously described, we assessed the specificity of the serum autoantibody by performing peptide/antigen competition assay, in which the serum was spiked with the target protein or peptide. The serum bound to tau eliminated the tau band in the Western blot (see Fig. 1) while the band of MAP-2 or MBP were present and not affected. The serum bound to MAP-2 eliminated the MAP-2 band in the Western blot while the band of tau or MBP was present. The serum bound to MBP eliminated the MBP band in the Western blot while the bands of tau and MAP-2 were present. These results indicate that each autoantibody in the serum was specifically neutralized by its target protein in serum sample and was no longer available to bind to the epitope present in the protein on the Western blot. This confirmed that the assay used in this study, was specific and accurately determined autoantibodies against tested proteins in serum samples.

To detect autoantibodies in serum, we probed Western blots with individual serum samples. A total of 30 human serum samples (20 veterans with GWI and 10 non-veteran symptomatic low-back-pain controls) underwent measurement of the levels of the serum circulating IgG-class autoantibodies against nine neuronal- and glial- specific proteins. Table 2 lists the number of GWI cases who were exposed to chemical and environmental exposures. It shows that 14 cases (70%) used PB as a prophylaxis against possible exposure to nerve agents and nine cases reported being exposed to the nerve agent sarin. In addition, a total of eight cases reported receiving notification from the Department of Defense (DOD) that they were potentially exposed to sarin and other chemicals due to their proximity to the Khamisiyah, Iraq underground weapons depot where a chemical weapons cache was destroyed in March 1991 (US DOD, 2002). Eight cases reported exposure to depleted uranium. All of the cases reported exposure to one or more insecticides or a mixture of pesticides including organophosphates, carbamates,

pyrethroids and organochlorines. Eleven cases used the insect repellent DEET. All cases underwent environmental and other exposures listed in Table 2. Other chemicals that the cases reported exposure to included oil well fires, sand, tent heaters, jet fuel, and solvents. Some veterans reported exposure to malaria and 18 reported being vaccinated. Serum from GWI cases showed significantly increased levels of autoantibodies against all cytoskeletal proteins except those against S-100B compared to non-veteran symptomatic (low back pain) controls (Table 3). Due to the gender differences between the cases and controls, analyses were also run with just the males in the groups. Although there was only a small number of males (*n* = 4) in the control group which could be problematic in this type of analysis, results of this comparison showed a very similar pattern of significant differences in all autoantibodies (GFAP *p* < 0.001; Tau *p* < 0.001; MAP *p* < 0.002; MAG *p* < 0.001; PNF *p* < 0.006; Tubulin *p* < 0.003; MBP *p* < 0.01; S-100B *p* = 0.31). The majority of GWI serum reacted intensely to neural proteins, while most control serum showed a weak or no reaction. Fig. 1a and b present Western blots results from three representative GWI cases and three controls. The levels of serum autoantibodies in GWI cases and controls to neural-specific proteins expressed as mean values \pm SD of triplicate assays of optical density arbitrary units normalized to total serum IgG optical density ranged from 0.30 for S-100B and 4.09 for GFAP for the cases compared to 0.30 and 0.62, respectively for controls are listed in Table 3 and shown in Fig. 2. The percentage of autoantibodies against neural proteins of cases compared to controls (in descending order) were: CaMKII, 927, GFAP 660, Tau 483, Tubulin 441, MAG 360, MBP 250, NFP 245, MAP-2 230, S-100B 103. Fig. 3 presents the mean values \pm SD (*p* < 0.001) of fold increase of autoantibodies against neural proteins for the cases compared with the controls. Serum from controls had no or low levels of circulating autoantibodies to nervous system-specific biomarkers. Autoantibodies against CaMKII were more predominant in the cases' serum than in controls' serum (Fig. 3).

Fig. 4 shows that Tubulin and GFAP had the highest values in the GWI cases compared with the controls. Pairwise correlations among the nine autoimmune biomarkers were significant only for the pair Tau and MBP. When comparing the correlation between each pair, only tau and MBP were significantly linearly correlated to each other (Fig. 5). Fig. 5 shows that the control values of those two biomarkers were < 1 optical density unit, whereas GWI cases had values strongly linearly correlated with each other such that on average tau was elevated up to 10 times higher than controls in some GWI cases, and MBP was also elevated up to 5 times higher for the same cases vs the controls.

Finally, when each biomarker was compared separately between individual cases and controls for potential fold-increase cut-points to discriminate the groups, results indicated that tubulin values had some of the highest-fold increased values in the individual GWI cases compared with the individual control values although only 60% of the individual cases (*n* = 12) showed that effect (Fig. 6a). However, in 9 (out of the 20) cases tubulin values were elevated by a factor of 3 to 9-fold higher than the controls. In Fig. 6b, GFAP was elevated the most in cases compared to controls. In fact, GFAP was higher in all of the cases compared with all of the controls with 20 out of 20 cases having 2 to 7 fold higher

Table 3
Statistical analysis of the levels^a of serum autoantibodies (AA) in controls^b and GWI cases^b to neural-specific proteins.

	NFP	Tau	Tubulin	MBP	MAG	MAP2	GFAP	S-100B	CaMKII
Cases	1.42 \pm 0.24	2.52 \pm 0.31	3.48 \pm 0.78	1.75 \pm 0.30	1.44 \pm 0.28	2.18 \pm 0.29	4.09 \pm 0.33	0.30 \pm 0.08	1.02 \pm 0.20
Mean \pm SD									
Controls	0.58 \pm 0.09	0.60 \pm 0.09	0.79 \pm 0.11	0.70 \pm 0.11	0.40 \pm 0.04	0.086 \pm 0.09	0.62 \pm 0.11	0.29 \pm 0.04	0.11 \pm 0.03
Mean \pm SD									
<i>p</i> values	0.02	0.0001	0.001	0.001	0.007	0.002	0.00001	0.40	0.015

^a The results are expressed as mean values of \pm triplicate assays of optical density arbitrary units normalized to IgG optical density as fold of healthy controls.

^b Values from cases were compared to the control group using *t*-tests; most were highly significant *p* < 0.001 (2-sided), except for S-100B that was not significantly different from controls. Cases were significantly different from controls with respect to gender *p* < 0.05 but not with respect to age.

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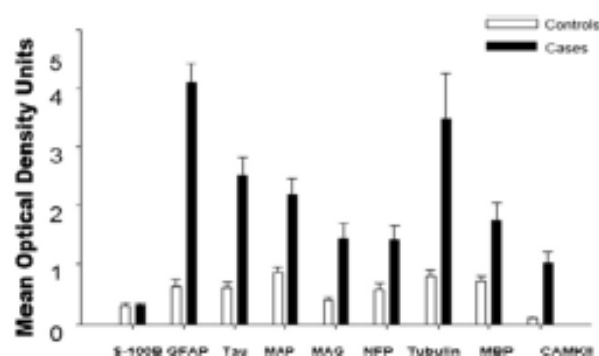


Fig. 2. Mean autoantibodies against neural proteins from cases and controls expressed in mean optical density units.

value than the control mean. Thus GFAP values completely distinguished the cases from the controls. GFAP values did not overlap in cases vs controls in this small sample; however, the separation in the ranges was small relative to the substantial standard deviations. In Fig. 6c, tau was higher than controls in 18 cases and 50% of the cases had double the value of tau compared with the controls. In Fig. 6d, MAP was higher than the controls in 15 cases and 75% of the cases had a 0.5 to 11-fold higher value than the controls. In Fig. 6e MAG was higher than controls in 15 cases and 75% of the cases had up to a 10-fold higher value than the controls. In Fig. 6f NFP was higher than controls in only 50% of the cases ($n = 10$) and they showed 0.5 to 11-fold higher values than controls. MBP was higher than controls in 12 cases and 60% of the cases were higher than controls with 2 to 5-fold higher values than controls. CAMKII was higher than controls in 16 cases and 50% of the cases had a 3 to 30-fold higher value than the controls. S100B values were not statistically significant as the values overlapped with cases and controls.

4. Discussion

This pilot study reports significantly elevated levels of autoantibodies against neurotypic- and gliotypic-specific proteins in serum from a sample of 20 veterans with GWI and 10 non-veteran symptomatic (low back pain) controls with musculoskeletal symptoms rather than CNS symptoms. The increased levels in GWI cases compared to controls ranged from 9.27 fold for CaMKII to 6.6 fold for GFAP to 2.45 fold for neurofilaments. Autoantibody levels against S-100B were not different in GWI cases than controls (1.03 fold) consistent with its neural protective role and in agreement with presence of chronic injury and

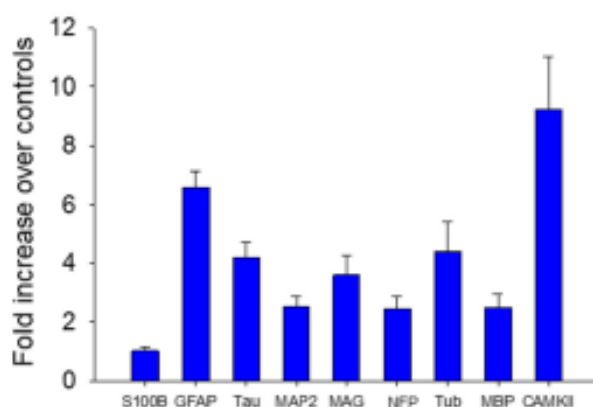


Fig. 3. Folds increase of autoantibodies against neural proteins from cases relative to controls.

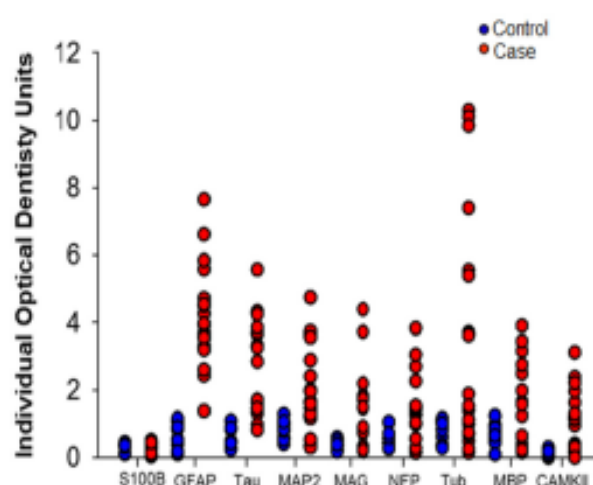


Fig. 4. The levels of autoantibodies of neural proteins of GW cases and of controls expressed as optical density units.

absence of acute brain injury in veterans with GWI (Zurek and Fedora, 2011; Diaz-Arastia et al., 2014; Stalnacke et al., 2006, 2004; Coch and Leube, 2016). Previous studies, using animal models of GWI, showed that exposure to the neurotoxins that were present in the GW environment, caused deficits in behavioral outcomes that were accompanied by neuronal and glial degeneration (Abdel-Rahman et al., 2001, 2002a,b, 2004a,b; Abou-Donia et al., 2000, 2001, 2004). Following neurodegeneration, there is accumulation of cellular neurological waste products or debris such as misfolded or hyper-phosphorylated proteins that form toxic stable aggregates (Nedergaard, 2013; Edgar et al., 2004). This extracellular debris send damage signals that cause the CNS immune cells – microglia to become activated and act as profound antigen presenting cells that secrete pro-inflammatory cytokines (IL-1b, TNF- α and IL-6) and mediators (reactive oxygen species, ROS) resulting in the recruitment of T-lymphocytes (Milligan and Watkins, 2009; Banks and Lein, 2012). Multiple exposures to these waste proteins can cause microglia and astrocytes to become primed to react more strongly after each subsequent exposure (Watkins and Maier, 2003). This can result in a persistent neuroimmune response and chronic neuroinflammation contributing to chronic health symptoms, such as those seen in GW veterans (Johnson et al., 2016; Milligan and Watkins, 2009; Maier and Watkins, 1998; Watkins and Maier, 2003). These waste proteins are eventually released into circulation due to defects in the

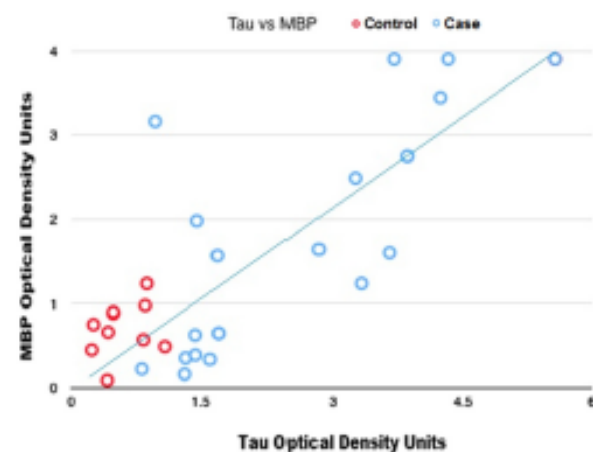


Fig. 5. Paired correlations of Tau and MBP optical density levels in cases relative to controls.

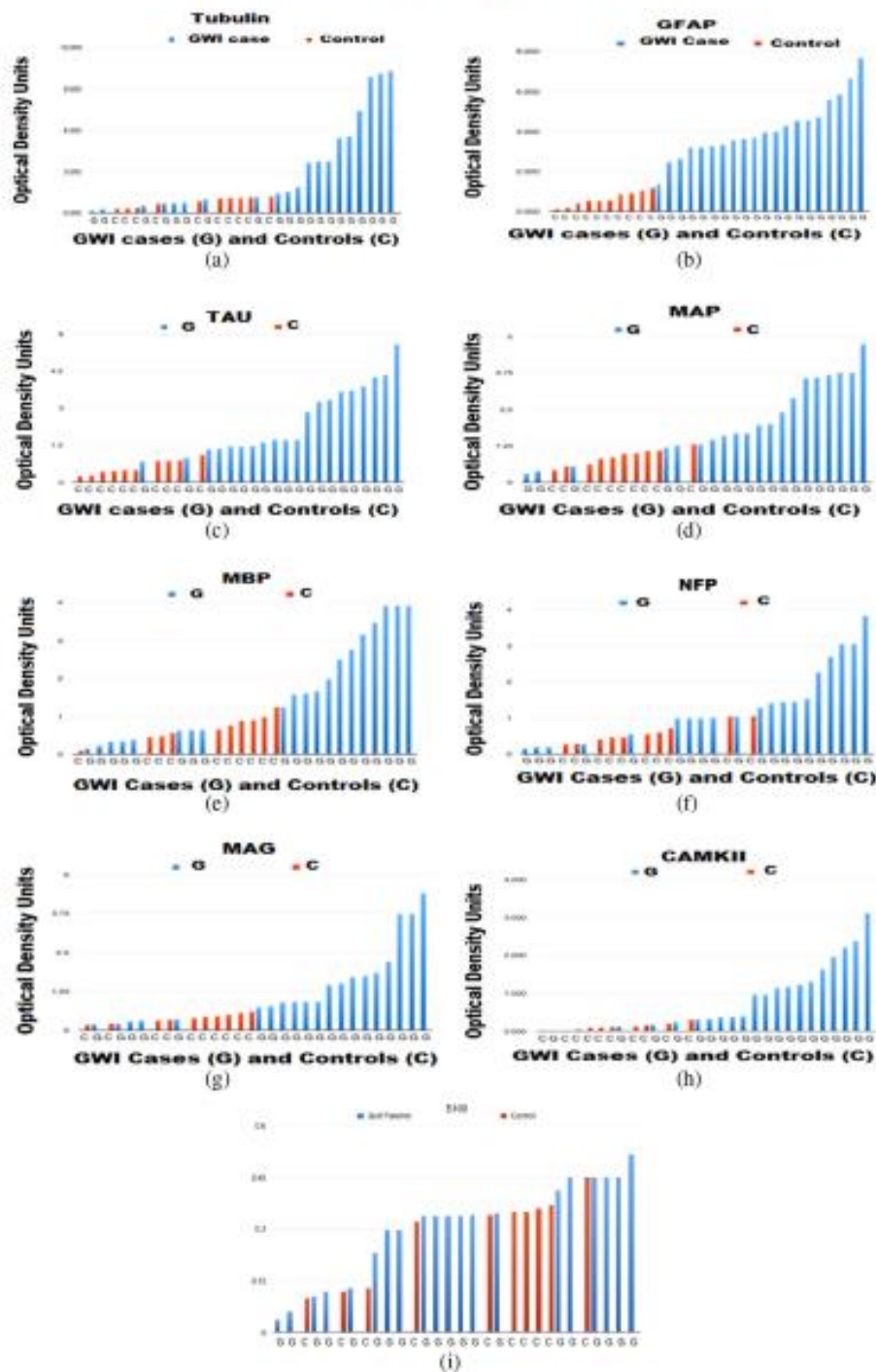


Fig. 6. a) Tubulin levels were higher than all controls in 12/20 cases. b) GFAP levels were higher than all controls in 20/20 cases. c) Tau levels were higher than all controls in 17/20 cases. d) MAP levels were higher than all controls in 15/20 cases. e) MBP levels were higher than all controls in 12/20 cases. f) NFP levels were higher than all controls in 10/20 cases. g) MAG levels were higher than all controls in 15/20 cases. h) CAMKII levels were higher than all controls in 16/20 cases. i) S100B levels overlap with cases and controls.

brain-blood barrier induced by astrocyte alterations. Waste proteins in the brain ultimately reach the liver through a mechanism known as the "glymphatic system" where they are degraded (Nedergaard, 2013). However, the released proteins that could serve as markers of injury are present in the short-term and cannot be used as biomarkers in the case of chronic GWI (Zurek and Fedora, 2011; Diaz-Arastia et al.,

2014). Thus detection of autoantibodies can serve as surrogate markers for these circulating waste proteins as described in this study.

The highest increase in autoantibodies was against CaMKII which was 9.27 times higher than that of controls followed by GFAP which was 6 times higher than controls. This result is consistent with the veterans' exposure during their deployment to the Gulf War to

organophosphorus compounds such as pesticides, and the nerve agent sarin that have been shown to increase the activity and mRNA expression of CaMKII (Patton et al., 1983, 1985, 1986; Gupta et al., 1998; Barbier et al., 2009) as well as enhanced CaMKII-induced phosphorylation of NFP, tubulin (Serrano et al., 1986) and tau activity leading to the aggregation, deregulation and accumulation of NFP (Abou-Donia et al., 1993; Norgren et al., 2003) and tubulin in the axon (Abou-Donia, 1993; Jensen et al., 1992; Gupta et al., 2000; Grigoryan and Lockridge, 2009). Aggregated neurofilaments result in slowing of axonal transport as has been illustrated in GW-relevant animal and cell neurotoxicant models (Gupta et al., 1997; Reagan et al., 1994; Terry et al., 2012; Gao et al., 2016; Edgar et al., 2004). GW-relevant exposure models have also been associated with astrocyte activation (Zakirova et al., 2015; Ojo et al., 2014).

Neuronal proteins studied in this pilot analysis represented various anatomical regions of the neuron with distinct functions which can be instructive with regard to the pathobiology of GWI (Lapadula and Abou-Donia, 1992). All of the proteins used are involved in axonal structure and function and are released as products of neural degeneration of various regions of the neuron. MAP-2 is present in the dendrites; CaMKII, tau, tubulin, and neurofilament proteins are located in the axon; myelin basic protein (MBP) and myelin associated glycoprotein (MAG) are an integral part of myelin (McMurray, 2000). Furthermore, the central nervous system-specific glial protein, GFAP and S-100B are secreted by astrocytes after neuronal injury (McMurray, 2000). Following axonal and myelin degeneration, neuronal and glial proteins are released and once in circulation, activated lymphocytes, B and T cells lead to the formation of autoantibodies against these proteins (Schwartz and Shechter, 2010a,b).

Increased autoantibodies against nervous system-specific proteins leads to structural consequences in various regions as follows: increased autoantibodies against neurofilaments proteins, tau, CaMKII and tubulin are indicative of axonal degeneration; increased autoantibodies against MAG and/or MBP suggest demyelination; increased autoantibodies against MAP-2 suggest dendritic degeneration; increased autoantibodies against GFAP suggest astrogliosis, and the low or no-increased levels of autoantibodies against S-100B is consistent with chemical-induced brain injury (Zurek and Fedora, 2011; Diaz-Arastia et al., 2014; Stalnacke et al., 2006, 2004). The linear correlation pattern of tau and MBP in this study suggests an important potential effect of axonal degeneration followed by demyelination that would correspond with prior neuroimaging studies in neurotoxicant exposed GW veterans (Heaton et al., 2007; Chao et al., 2010). Furthermore, these structural changes of the nervous system lead to functional alterations. Hence axonal degeneration in the cerebral cortex leads to: motor and sensory abnormalities, ataxia, deficit in posture, locomotion, and skilled fine motor movements (fingers, speech, facial expression) and weakness; degeneration of the limbic system including the hippocampus leads to: learning and memory deficits, and neurobehavioral (mood, emotion and judgment) abnormalities; increased autoantibodies against MAP-2 suggests damage to the dendrite-rich Purkinje cells in the cerebellum resulting in: gait and coordination abnormalities, staggering gait and ataxia (McMurray, 2000; Abou-Donia, 2015). Increased autoantibodies against GFAP indicate astrogliosis and potential neuroinflammation and/or glial scarring. GFAP contributes to white matter architecture, myelination and blood brain barrier (BBB) integrity (O'Callaghan and Sriram, 2005; Amourette et al., 2009; Lamproglou et al., 2009). Consequently, blood levels of GFAP in healthy individuals are very low. GFAP levels were higher in GW cases and completely discriminated between the cases and controls in this study. This is particularly relevant because disorders with higher levels of GFAP include memory disorders such as Alzheimer's and vascular dementia that have significant axonal neurodegeneration and neuroinflammation (Mecocci et al., 1995). Increased autoantibodies against S-100B suggest traumatic brain damage and can help to differentiate between acute and chronic brain injury (Stroick et al., 2006; Stalnacke et al., 2006, 2004; Zurek and Fedora,

2011; Diaz-Arastia et al., 2014; Coch and Leube, 2016). Their lack of increase in this study suggests against acute traumatic brain injury in veterans with GWI.

Important mechanistic clues from animal and cell studies of these GW-relevant neurotoxicants have shown deficits in axonal transport, as well as aberrations in neurofilaments and microtubules, which are the structural railways for axonal transport (Gupta and Abou-Donia, 1995a, b; Gearhart et al., 2007; Grigoryan and Lockridge, 2009; Prendergast et al., 2007; Jiang et al., 2010). Mitochondria are also delivered by axonal transport to provide the energy required to power the biochemical reactions necessary for the functioning of the axon and have shown altered functioning in GW-relevant neurotoxicant models (Middlemore-Risher et al., 2011). GW-relevant chronic low-level organophosphate exposure has also been associated with mitochondrial compromise from oxidative stress induction and with neuroinflammation resulting in cell damage or cell death resulting in debris of waste proteins in the extracellular spaces (Laetz et al., 2009; Kaur et al., 2007; Banks and Lein, 2012). In fact, one hypothesis of GWI suggests that mitochondrial damage and oxidative stress in the brain and the periphery have caused the chronic symptoms of GWI; notably, increased autoantibodies were expressly cited among objective markers and mediators in this model (Golomb et al., 2014; Golomb, 2012; Koslik et al., 2014).

Another hypothesis of GWI suggests that the neurotoxicants acted synergistically to create a self-perpetuating neuroinflammatory state, which in turn has an ongoing negative impact on brain cells including neurons (microtubules, motor proteins, mitochondria) and glia (microglia, astrocytes, oligodendrocytes) and blood-brain barrier function (O'Callaghan and Sriram, 2005). Clinical studies have also found consistent results with GW veteran cohorts who showed impaired cognitive functioning and reduced volume and altered white matter microstructural integrity on MRI in OP pesticide, sarin nerve agent and PB pill exposed cohorts (White et al., 2016; Sullivan et al., 2013; Chao et al., 2010; Heaton et al., 2007; Proctor et al., 2006; Sullivan et al., 2003). These prior results suggest clear CNS alterations in neurotoxicant-exposed GW veterans which correlated with behavioral outcomes that are related to neurodegeneration and perhaps with both a chronic neuroinflammatory and Mitochondrial/OS hypothesis.

The only other study that we are aware of that compared CNS autoantibodies in GW veterans compared MBP and striated and smooth muscle antibodies and reported higher MBP and muscle antibodies in veterans with GWI when compared with controls (Vojdani and Thrasher, 2004). The current study validates the prior MBP findings and expands on those findings with a larger panel of 8 additional CNS autoantibody markers. Collectively, these findings suggest that alterations in white matter as evidenced by circulating autoantibodies to MBP appear to be associated with GWI. This finding corresponds with both leading hypotheses for GWI given that white matter alterations can be associated with oxidative stress and neuroinflammation as a result of glial activation and signaling of both proinflammatory cytokines and oxidative stress (Milligan and Watkins, 2009). The additional finding of this study that higher Tau autoantibody levels were significantly linearly correlated with higher MBP autoantibody levels in GWI cases suggests that axonal degeneration may be occurring before demyelination in veterans with GWI and warrants further more conclusive study to distinguish it from the more myelin-specific toxic leukoencephalopathies (Schmahmann et al., 2008; Filley, 2013). These findings also correspond with MRI findings of differences on both white and gray matter brain volumes in neurotoxicant-exposed GW veterans (Heaton et al., 2007; Chao et al., 2010, 2011, 2014, 2016). These findings also clearly suggest that glia and astrocytes in particular should be further studied in GWI given significantly higher levels of GFAP in the GWI cases that correspond with prior animal models of GWI (Abdel-Rahman et al., 2001, 2002a, 2002b, 2004a, 2004b; Abou-Donia et al., 2000, 2001, 2002, 2004; Zakirova et al., 2015; Ojo et al., 2014) and with recent studies illustrating the ability of astrocytes to donate mitochondria to damaged neurons (Hayakawa et al., 2016).

4.1. Limitations and future directions

This study, like all studies has important limitations. Although the present pilot study can serve as a proof-of-concept it has a small sample size and non-matched subject groups for age, gender and for CNS symptoms. This is particularly important as it has also been shown that CNS autoantibodies have been reported to be age-related in animal models (Lal and Forster, 1988). In addition, the convenience comparison group utilized in this study had musculoskeletal symptoms and not CNS symptoms therefore, it remains to be shown that these CNS autoantibody markers can clearly distinguish between GWI cases and additional groups with CNS specific symptoms. However, the strong results including 9-fold higher levels of CAMKII, 6-fold higher levels of GFAP and 4-fold higher levels of tau and tubulin that were presented in this study warrant further research for a blood-based objective marker of GWI in larger, well-characterized veteran cohorts. These results suggest a possible new avenue for further development of an objective biomarker of GWI. The identification of this small panel of neural-specific autoantibody biomarkers in GWI shows promise for further validation in larger study samples that are more carefully matched for subject demographics (particularly age), different types of control groups (i.e. healthy and CNS symptomatic groups) and that classify cases by both the CDC and the more specific Kansas GWI criteria which also specifies the time period of deployment which may be relevant to particular OP and other deployment-related exposures (Steele, 2000; Fukuda et al., 1998). Future directions will be to compare these CNS autoantibody markers with specific behavioral outcomes including cognitive performance and brain imaging of gray and white matter volume and microstructural integrity to further validate these suspected brain-immune-behavioral outcomes.

5. Conclusions

In conclusion, in this pilot study GWI was significantly associated with 2–9 fold increased serum autoantibodies against 8 neuronal and glial-specific proteins (CaMKII, GFAP, Tau, Tubulin, MAG, MBP, NFP, MAP-2) and not with a marker of more acute damage (S-100B). The autoantibodies that were found here to be elevated in GWI, targeted proteins/antigens that play critical roles in the structure and function of the neuron including axonal transport and myelination. Many of them are explicit markers for neurodegenerative disorders, consistent with axonal and myelin degeneration of myelinated neurons and with astrogliosis, cell signaling and neuroinflammation. These same proteins have been shown to be affected in other clinical groups and animal models with similar organophosphate and carbamate exposures (Abou-Donia et al., 2013, 2014). These results validate prior reports of increased MBP autoantibodies in GWI cases and suggest that oligodendrocyte signaling, glia and white matter alterations should continue to be further studied in GWI and validated with health symptom and behavioral outcomes (Vojdani and Thrasher, 2004). The results also indicate that veterans with GWI may be continuing to show brain neuronal degeneration and glial activation that would be consistent with recent reports of chronically persistent and in some cases worsening health of these veterans (Smith et al., 2013; Ozakinci et al., 2006; Li et al., 2011; Kang et al., 2009; Dursa et al., 2016; White et al., 2016). These results suggest a possible avenue for further development of a panel of objective biomarkers of GWI upon further validation in larger study samples that are more carefully matched for subject demographics.

Conflict of interest statement

The authors report no relationships that could be construed as a conflict of interest.

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IRB NOTIFICATION OF CONTINUING REVIEW APPROVAL

Continuing Review ID: CR012_Pro00003202
Principal Investigator: Mohamed Abou Donia
Protocol Title: Nervous System Injury
Duke University School of Nursing Office of Research Affairs Small Grant Award
Sponsor/Funding Source(s): Program

Federal Funding Agency ID: GW 140140P2

Date of Declared Concordance with federally funded grant, if applicable: N/A

The Duke University Health System Institutional Review Board for Clinical Investigations has conducted the following activity on the study cited above:

Activity:	Continuing Review	Review Type: Expedited
Review Date:	10/9/2017	
Issue Date:	10/9/2017	
Anniversary Date:	11/5/2017	
Expiration Date:	11/5/2018	

DUHS IRB approval encompasses the following specific components of the study:

Protocol, version/date:	--
Summary, version/date:	--1/4/2017
Consent form reference date:	--10/9/2017
Investigator Brochure, version/date:	--

Pediatric Risk Category: -- 45CFR46.404 and 21 CFR 50.51 as applicable
Other: --

The DUHS IRB has determined the specific components above to be in compliance with all applicable Health Insurance Portability and Accountability Act ("HIPAA") regulations.

This study expires at 12 AM on the Expiration Date cited above. At that time, all study activity must cease. If you wish to continue specific study activities directly related to subject safety, you must immediately email Jody Power at jody.power@duke.edu or call the IRB Office at 668-5111 and follow the instructions to reach the IRB Chair on call. Continuing review submissions (renewals) must be received by the DUHS IRB office 60 to 45 days prior to the Expiration Date.

No change to the protocol, consent form or other approved document may be implemented without first obtaining IRB approval for the change. Any proposed change must be submitted as an amendment. If necessary in a life-threatening situation, where time does not permit your prior consultation with the IRB, you may act contrary to the protocol if the action is in the best interest of the subject. You must notify the IRB of your action within five (5) working days of the event.

The Duke University Health System Institutional Review Board for Clinical Investigations (DUHS IRB), is duly constituted, fulfilling all requirements for diversity, and has written procedures for initial and continuing review of human research protocols. The DUHS IRB complies with all U.S. regulatory requirements related to the protection of human research participants. Specifically, the DUHS IRB complies with 45CFR46, 21CFR50, 21CFR56, 21CFR312, 21CFR812, and 45CFR164.508-514. In addition, the DUHS IRB complies with the Guidelines of the International Conference on Harmonization to the extent required by the U. S. Food and Drug Administration.



DUHS Institutional Review Board
2424 Erwin Rd | Suite 405 | Durham, NC | 919.668.5111
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